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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 9/127 — A1

(11) International Publication Number: WO 94/22428

(43) International Publication Date: 13 October 1994 (13.10.94)

(21) International Application Number: PCT/US94/03326

(22) International Filing Date: 28 March 1994 (28.03.94)

(30) Priority Data:
08/040,537 30 March 1993 (30.03.93) US

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(81) Designated States: AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR REDUCING THE INDUCTION OF VIRUSES USING TOPICALLY APPLIED ANTIOXIDANTS

(57) Abstract

A method for treating an individual infected with an inducible virus, such as, the HIV, herpes simplex, Epstein-Barr, or hepatitis B viruses, is provided in which at least one antioxidant or liposomes containing at least one antioxidant or mixtures thereof are topically administred to the infected individual. The antioxidant or liposomes containing an antioxidant are effective in reducing induction of the virus by reactive oxygen species resulting from skin damage caused by such agents as UV and ionizing radiation.

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METHOD FOR REDUCING THE INDUCTION OF VIRUSES USING TOPICALLY APPLIED ANTIOXIDANTS

FIELD OF THE INVENTION

This invention relates to viral diseases which are inducible by reactive oxygen species and, in particular, to methods for reducing such induction.

BACKGROUND OF THE INVENTION

I. Inducible Viral Diseases

Various viral diseases are known which are inducible by reactive oxygen species (ROS), i.e., free oxygen radicals or compounds which can become involved in free radical reactions. Clinically important examples of these viruses include the human immunodeficiency virus (HIV), the herpes simples virus (HSV), the Epstein-Barr virus (EBV), and the hepatitis B virus (HBV). Examples of ROS which can induce these viruses include superoxide radicals, hydroxyl radicals and hydrogen peroxide. Damage to skin by radiation in the form of ultraviolet light (UV) or ionizing radiation can create reactive oxygen species in living tissue. In addition, inflammatory and immunological reactions in response to skin damage are known to create ROS.

The human immunodeficiency virus type I, although an RNA virus, is similar to other human pathogenic DNA viruses, e.g. herpes simplex virus type I, Epstein-Barr virus, and hepatitis B virus, in that it is able to latently infect specific cells and lay dormant for long periods before a stimulus leads to a pathogenic infection. Garcia-Blanco M, Cullen B: Molecular basis of latency in pathogenic human viruses. Science, 254:815-820, 1991; Bednarick D, Folks T: Mechanisms of HIV-1

latency. AIDS, 6:3-16, 1992. Localization of latent HIV in the skin is of concern because as discussed above, the latent virus can be activated by environment factors, including ultraviolet light and X-irradiation, which create ROS. Legrand-Poels S, Vaira D, Pincemail J, Van de Vorst A, Pietter J: Activation of human immunodeficiency virus type I by oxidative stress. AIDS Research and Human Retroviruses, 6:1389-97, 1990; Zmudzka B, Beer J: Activation of human immunodeficiency virus by ultraviolet radiation. Photochemistry and Photobiology, 52:1153-1162, 1990; Nokta M, Belli J, Pollard R: X-irradiation enhances in vitro human immunodeficiency virus replication. Proc. Soc. Exper. Biol. Med., 200:402-408, 1992.

Activation of latent human viruses in skin is clinically relevant because solar UV exposure and other damaging agents from the environment are virtually unavoidable, and cutaneous lesions in HIV and HSV infected patients are frequently found on exposed areas of the skin. HIV seropositive patients often have higher solar and artificial UV exposure than matched controls, due to their mistaken belief that a suntan will improve their health. In addition, UV is a component of therapy for many dermatological diseases, and infected patients may thus receive UV exposure in the course of therapy.

Transgenic mice with the HIV LTR controlling the luciferase gene were treated with ROS, specifically hydrogen peroxide, and activated gene expression was observed. Morrey J, Bourn S, Bunch T, Sidwell R, Rosen C: HIV-1 LTR Activation Model: Evaluation of various agents in skin of transgenic mice. Journal of Acquired Immune Deficiency Syndromes, 5:1195-1203, 1992. Increased transcription was also observed after irradiation with UV and UV-plus-psoralen (PUVA). Morrey J, Bourn S, Bunch T, Jackson M, Sidwells R, Barrows L, Daynes R, Rosen C: In vivo activation of human immunodeficiency virus type 1 long terminal repeat by UV type A (UV-A) plus psoralen

and UV-B light in the skin of transgenic mice. Journal of Virology, 65:5045-5051, 1991; Vogel J, Cepeda M, Tschachler E, Napolitano L, Jay G: UV activation of human immunodeficiency virus gene expression in transgenic mice. Journal of Virology, 66:1-5, 1992. Similarly, transgenic mice with the HIV LTR linked to the lacZ gene have been UV irradiated and an increase in ß-galactosidase has been detected in skin. Cavard C, Zider A, Vernet M, Bennoun M, Saragosti S, Brimber G, Briand P: In vivo activation by ultraviolet rays of the human immunodeficiency virus type 1 long terminal repeat. Journal of Clinical Investigations, 86:1369-1374, 1990.

Prior to the present invention, no method for directly preventing cutaneous induction of viruses by ROS has been available. Studies have focused on the systemic use (oral or parenteral) of antioxidants, i.e. agents which remove or prevent the formation of ROS, in AIDS patients. In addition, the studies have been concerned only with treating a disease state such as AIDS, and not with preventing viral induction from environmental exposure. See, for example, Harakeh S, Jariwalla R and Pauling L: Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. Proceeding of the National Academy of Sciences, U.S.A., 87:7254-7249, 1990. Moreover, expert opinion has been that little benefit would be derived from systemic uses of antioxidants. Drs. Barry Halliwell and Carroll Cross of the University of California, Davis, wrote in their paper entitled "Reactive Oxygen Species, Antioxidants and Acquired Immunodeficiency Syndrome" Archives of Internal Medicine, 151:29-31, 1991: "In our minds, the evidence does not yet justify testing of nonphysiologic scavengers of reactive oxygen species in humans, especially as they can sometimes have harmful effects" (emphasis theirs). None of these studies on the systemic use of antioxidants has made any suggestion to

test the effects of topically applied antioxidants to prevent viral induction.

II. Antioxidants and Liposomes Containing Antioxidants

Antioxidants are a natural component of skin, and sun exposure depletes antioxidant activity in both epidermis and dermis. Shindo Y, Witt E, Packer L: Antioxidant defense mechanisms in murine epidermis and dermis and their responses to ultraviolet light. Journal of Investigative Dermatology, 100:260-265, 1993. Cosmetic lotions containing antioxidants, specifically, vitamin E, at concentrations in the range of 1-5% w/v are commercially available.

Topical application of 5% lotions of freely dissolved antioxidants prior to UV irradiation has been reported to reverse chronic damage to skin, including wrinkling and skin cancer. See Bissett D, Chatterjee R, Hannon D: Photoprotective effect of superoxide-scavenging antioxidants against ultraviolet radiation-induced chronic skin damage in the hairless mouse. Photodermatology, Photoimmunology and Photomedicine, 7:56-62, 1990. Since the antioxidants used in this study were also UV sunscreens, the effect of the compounds may have been only as UV absorbers, not as antioxidants. Post-UV application of 100% α-tocopherol was reported to reduce sunburn-associated erythema. Trevithick J, Xiong H, Lee S, Shum D, Sanford S, Karlik S, Norley C, Dilworth G: Topical tocopherol acetate reduces post-UVB, sunburn-associated erythema, edema, and skin sensitivity in hairless mice. Archives of Biochemistry and Biophysics, 296:575-582, 1992. Other studies involving antioxidants and UV exposure include: Black H: Potential involvement of free radical reactions in ultraviolet light mediated cutaneous damage. Photochem. Photobiol., 46:213-221, 1987; and Law E., Lewis A: The effect of systemically and topically applied drugs on ultraviolet-induced erythema in the rat. British Journal of Pharmacology, 59:591-597, 1977.

Liposomes are microscopic vesicles composed of membranes normally prepared from amphipathic phospholipids. They may be unilamellar or multilamellar. Hydrophilic compounds may be sequestered in the internal spaces between the membranes and hydrophobic compounds may be included within the liposomal membranes. The interaction of vitamin E with model membranes has been studied through the use of liposomes. Govil G, Phadke R, Srivastava S: Physical/Chemical Studies of Vitamin E in Membranes. Lipid-Soluble Antioxidants, Ong A and Packer L (eds.), Birkhauser Verlag, Basel, Switzerland, 1992, pages 28-46. Liposomes have been shown to penetrate the skin's stratum corneum and administer their contents to the interior of the cells of the epidermis. Yarosh D, Alas L, Yee V, Oberyszyn A, Kibitel J, Mitchell D, Rosenstein R, Spinowitz A, Citron M: Pyrimidine dimer removal enhanced by DNA repair liposomes reduces the incidence of UV skin cancer in mice. Cancer Research, 52:4227-4231, 1992; Yarosh, U.S. Patents Nos. 5,077,211 and 5,190,762 and Yarosh, PCT Patent Publication No. WO90/00598, published January 25, 1990.

None of this prior work with antioxidants, liposomes, or liposomes containing vitamin E, however, has disclosed or suggested that such antioxidants or liposomes containing such antioxidants are able to reduce the induction of viruses by ROS.

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of this invention to provide methods for reducing the induction of inducible viruses by ROS. More particularly, it is an object of the invention to provide such methods which can be conveniently used by individuals infected with such viruses.

To achieve the foregoing and other objects, the invention provides a method for treating an individual infected with an inducible virus comprising topically administering at least one antioxidant or liposomes

containing at least one antioxidant or mixtures thereof to the infected individual in an amount effective to reduce induction of the virus by damage to the individual's skin which creates ROS.

Among the inducible viral infections which can be treated in this way are HIV (Type I and II), HSV (Type I and II), EBV, and HBV. Among the antioxidants which can be used either in unencapsulated form or incorporated in liposomes are vitamin C, vitamin E, probucol, and superoxide dismutase. Among the ROS-creating agents whose virus inducing effects can be reduced are UV and gamma or x-ray radiation.

The accompanying drawings, which are incorporated in and constitute part of the specification, illustrate the preferred embodiments of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the drawings and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates inhibition of UV induction of HIV by antioxidant liposomes. XPHIV-II cells were irradiated with 5 J/m^2 UV-C, incubated with liposomes containing vitamin E and C (circles) or liposomes containing vitamin E and probucol (squares), and CAT activity was measured.

Figure 2 illustrates inhibition of HIV activation in transgenic mice by antioxidant liposomes. Each of eight transgenic mice with the HIV LTR viral promoter sequence linked to the luciferase gene in their epidermis were irradiated with UV-B, and luciferase activity compared between placebo treated skin (set to 100%) and skin treated with antioxidant liposomes, i.e., either vitamin E and C liposomes or liposomes containing vitamin E and probucol. Each bar in Figure 2 represents the results for one of the transgenic mice.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to the treatment of viral diseases by the topical application of antioxidants or of liposomes containing antioxidants or of mixtures thereof. Either a single antioxidant or a mixture of antioxidants can be used in the practice of the invention.

As used herein, the term "antioxidant" is intended to embrace antioxidants per se, compounds (precursors) which upon topical application to the skin release or are converted to antioxidants per se, and compounds (derivatives) such as salts, esters, or anhydrides of antioxidants which either release or are converted to antioxidants upon topical application or which otherwise provide to the skin the effective antioxidant functionality of the invention. Similarly, the terms "α-tocopherol", "ascorbic acid", and "probucol" are intended to embrace these compounds per se and functional equivalents of the types described above with regard to the general term "antioxidant".

The antioxidants will normally be of the type which can inhibit lipid peroxidation, such as α-tocopherol (vitamin E). Other antioxidants scavenge ROS, such as superoxide dismutase or ascorbic acid (vitamin C). Antioxidants can also function by inhibiting the formation of ROS and by binding transition metal ions. A preferred antioxidant is α-tocopherol, which is normally found in cell membranes and is readily incorporated into liposome membranes. As discussed above, precursors and derivatives, such as physiologically acceptable salts of the antioxidants can be used in the practice of the invention. For example, derivatives which enhance the ability of unencapsulated antioxidant to penetrate the skin can be used if desired.

The antioxidants in either encapsulated or unencapsulated form may be applied to the skin in compatible vehicles, such as creams, ointments or

lotions. Upon application, the antioxidants must have the ability to inhibit or deactivate ROS, since antioxidants which have lost this activity during storage are no longer able to have an effect on virus induction.

An unexpected finding of the present invention is that liposome encapsulation of antioxidants allows the use of concentrations approximately one one-hundredth (1/100) or less of that of unencapsulated antioxidant. In addition, the proper combination of antioxidants in liposomes can increase the overall stability of the antioxidants. For example, the combination of vitamin C and vitamin E in the same liposome allows the vitamin C encapsulated in the aqueous phase to reduce oxidized vitamin E in the membrane and restore its antioxidant properties. Packer L: Interactions Among Antioxidants In Health and Disease: Vitamin E and Its Redox Cycle, Proceedings of the Society of Experimental Biology and Medicine, 200:271-276, (1991). Likewise, the UV absorbing vitamin E in the membrane surrounds the entrapped vitamin C and protects it from photodegradation.

The liposomes used in the practice of the invention preferably have the compositions disclosed in Yarosh, U.S. Patents Nos. 5,077,211 and 5,190,762, and Yarosh, PCT patent publication No. WO 90/00598, the relevant portions of which are incorporated herein by reference. In particular, pH sensitive liposomes composed of phosphatidyl choline, phosphatidyl ethanolamine, oleic acid, and cholesteryl hemisuccinate in a 2:2:1:5 molar ratio are preferred for the practice of the invention. The antioxidants are encapsulated in the liposomes using conventional techniques, with hydrophilic antioxidants being localized in the aqueous compartment and lipophilic antioxidants being localized in the liposomes' membranes.

When a mixture of antioxidants is to be administered using liposomes, the mixture can be formed by separately encapsulating individual antioxidants in liposomes, by

mixing the set of antioxidants prior to encapsulation, or by adding lipophilic antioxidants to the lipid membrane and encapsulating hydrophilic antioxidants in the aqueous phase.

The antioxidant liposomes are incorporated in a suitable vehicle for topical application. Since the liposomes are composed of lipids, the vehicle should be of the type in which lipids are not substantially soluble to avoid dissolving the liposome membranes, e.g., the vehicle should be water based and should not include detergents, emulsifiers, or lipid solubilizers such as sodium dodecylsulfate, Triton X-100, octylglucopyranoside, or ethylene glycol or its derivatives. Further, since environmental exposure and, in particular, UV light, can oxidize the lipids of the liposomal membrane, the lotion should not contain oxidizing agents and should be protected from environmental and, in particular, UV exposure prior to use. In the case of pH sensitive liposomes, the vehicle should be buffered to a pH above the pH at which the liposome membranes are destabilized, e.g., a pH of 7 or greater. Examples of suitable vehicles include the hydrogels HYPAN SS201 produced by Kingston Hydrogels, Dayton, New Jersey, and CARBOPOL-941 produced by B. F. Goodrich, Brecksville, Ohio. To form the vehicle, these hydrogels are neutralized with triethanolamine and hydrated with phosphate buffered saline to a final concentration of 1.0% to 2.0% w/v.

Typical concentrations of unencapsulated antioxidants in creams, ointments or lotions are 10 to 50 mg/ml, or approximately 1% to 5% w/v. When the antioxidants are encapsulated in liposomes and the liposomes incorporated in a lotion, typical concentrations of the antioxidants in the lotions are 1 to 100 μg antioxidant per ml lotion, or approximately 0.0001% to 0.01% w/v. A typical dosage of the final composition for application to the skin whether of

unencapsulated antioxidant or encapsulated antioxidant is in the range from about 20 to about 100 μl of lotion per square centimeter of skin. Other concentrations and dosages can be used in the practice of the invention if desired.

Latent inducible viruses can be activated from many different cell types which they may infect, including keratinocytes of the skin, fibroblasts, and T-cells, which are the preferred target of the HIV virus. The signals for activation are derived in part from cis-acting elements within the ROS-exposed cell and in part from intercellular mediators produced by ROS-exposed cells which can travel to infected untreated cells and induce the virus.

The antioxidants or antioxidant liposomes of the invention should be able to reach skin cells upon which ROS act, e.g., the melanocytes and keratinocytes of the epidermis. In particular, keratinocytes, which comprise the majority of the cells of the epidermis, should be reached by the antioxidants. Langerhans cells which can be infected by viruses may also be an important target. Melanocytes may be a further important target, since these cells are often damaged by ROS and can generate signals for infiltration of neutrophils, and other cell types, which produce ROS.

The skin to which the antioxidants or antioxidant liposomes are applied will normally be human skin, although the invention can also be used to treat animals infected with inducible viruses. The antioxidants or antioxidant liposomes are preferably applied to the skin at or near the time of exposure to ROS.

The present invention can be used to treat individuals infected with a variety of inducible viruses now known or subsequently identified. For example, cytomegalovirus is in the herpes family and may be inducible by ROS, in which case, it can be treated using the present invention. Similarly, the invention can be

used to reduce induction by a variety of ROS now known or subsequently identified. The preferred applications of the invention are to HIV, EBV, HSV, and HBV viruses and to ROS created by UV and ionizing radiation. With regard to UV radiation it should be noted that the wavelengths of light which activate viruses are those in the range of 240-360 nm. The most effective wavelengths are those of UV-B light (280-320 nm), with UV-A light (320-360 nm) being less effective, except in the case of exposure in the presence of psoralen or its derivatives. UV-C (240-280 nm) is quite effective in activating latent viruses, especially HIV, but this wavelength is screened out from the earth's surface by the atmospheric ozone layer.

Without intending to limit it in any manner, the present invention will be more fully described by the following examples.

Example 1

Preparation of Antioxidant Liposomes

The lipid membrane of the antioxidant liposomes was composed of phosphatidylcholine, phosphatidyl-ethanolamine, oleic acid and cholesterol hemisuccinate (2:2:1:5 molar ratio). The vitamin E and C liposomes contained the four membrane components together with α-tocopherol in a 2:2:1:5:4 molar ratio, and magnesium ascorbic phosphate encapsulated in the aqueous phase at 1.5% w/v. The probucol and vitamin E liposomes contained the four membrane components together with α-tocopherol and probucol in a 2:2:1:5:4:4 ratio. The amount of each component was calculated so that the final concentration of phospholipids in the aqueous solution of liposomes prior to gel filtration was 10mM.

The four membrane components were mixed together with vitamin E and, when appropriate, probucol, in a volume of chloroform approximately equal to the final volume of aqueous solution of liposomes prior to gel filtration. Octylglucopyranoside was added so that its final concentration in the aqueous solution was 25 mM.

The chloroform was then removed by evaporation on a rotary evaporator with heating at 37°C, and the final traces of chloroform were removed by vacuum for 30 min.

An aqueous solution was then added to the dried components and the resulting solution rotated for 30 minutes at room temperature until all the components were in solution. In the case of vitamin E and C liposomes, the aqueous solution contained 1.5% (w/v) magnesium ascorbic phosphate. In the case of vitamin E and probucol liposomes, the aqueous solution was phosphate buffered saline. The aqueous solution was then dialyzed overnight and refrigerated against a ten fold larger volume of phosphate buffered saline with one change of dialysis buffer. The resulting liposome solution was then passed through a SEPHADEX G-75 gel filtration column to separate the liposomes from unencapsulated material, and the turbid liposomes collected, and stored refrigerated.

The concentration of the antioxidant components was measured by chloroform phase separation and high pressure liquid chromatography. A sample of liposomes was mixed with an equal volume of chloroform. The chloroform layer was removed, the chloroform evaporated, and the extracted lipophilic antioxidants (e.g. vitamin E and/or probucol) were resuspended in methanol. These antioxidants were quantitated by injecting onto a C_{18} reverse-phase HPLC column with elution by 100% methanol and detection at 280 nm. The aqueous layer containing hydrophilic antioxidants (e.g. vitamin C) were reduced with 10 mM dithiothreitol and injected onto the same HPLC column with elution by 10% methanol, 90% 10 mM ammonium formate pH 4, with detection by 254 nm UV. The antioxidants were tested for their activity by their ability to prevent the UV-induced formation of thiobarbituric acid reacting substances in the lipid membrane, widely known in the literature as the TBARS assay. See, for example, Pelle E, Maes D, Padulo G, Kim E-K., and Smith W: <u>In Vitro</u>

Model to Assess Alpha-Tocopherol Efficacy. Annals of the New York Academy of Sciences, 570:491-494, 1989.

The vitamin E and C liposomes contained 561 μg vitamin C per ml of liposome solution and 60 μg vitamin E per ml of liposome solution. The vitamin E and probucol liposomes contained 186 μg probucol per ml of liposome solution and 87 μg vitamin E per ml of liposome solution.

Example 2

Inhibition of UV Induction of HIV by Antioxidant Liposomes

The ability of antioxidant liposomes to inhibit UV induction of HIV was tested by irradiating human cells with 5 J/m^2 UV-C and incubating post-irradiation with increasing concentrations of liposomes containing vitamin E and either probucol or vitamin C. The human cells (hereinafter referred to as XPHIV-II) carried the HIV LTR promoter linked to the chloramphenicol acetyltransferase (CAT) gene. UV irradiation of cell lines of this type has been shown to induce CAT gene expression. Zmudzka B, Beer J: Activation of human immunodeficiency virus by ultraviolet radiation. Photochemistry and Photobiology, 52:1153-1162, 1990.

XPHIV-II Cell Line. SV40-transformed excision-repair deficient human fibroblast strain XP12BE from a group A xeroderma pigmentosum patient was obtained from the Coriell Institute for Medical Research (Camden, NJ). It was transfected by the calcium phosphate method using plasmid pHIVcatSVneo from the Medical College of Virginia, Richmond, Virginia. XPHIV-II was produced by selection with 800 μg/ml geneticin and pooling of resistant colonies.

UV-irradiation and Cell Culture. Cells were cultured with Dulbecco's modified Eagle's medium prepared from powder (DME; GIBCO, Grand Island, NY) with 10% newborn calf serum (Irvine Scientific, Santa Ana CA), 50 μg/ml gentamicin and 0.5 μg/ml amphotericin B in 60 mm

dishes to 80% confluence. They were irradiated without medium with UV-C from a Phillips G15T germicidal lamp emitting predominantly 254 nm light at a fluence rate of 1 J/m^2/sec, as monitored by a UVX digital radiometer (Ultraviolet Products, San Gabriel, CA). The cells were refed with 5 ml media containing 4% serum and either 0, 5, 25, 100 or 250 μl of antioxidant liposomes, and incubated overnight at 37°C, whereupon CAT activity was determined as described below.

Chloramphenicol Acetyltransferase Assay. Extracts were prepared from cells harvested in 0.25 M Tris, pH 7.8 by three cycles of freeze-thawing and protein concentration was determined by the Bradford reaction. CAT gene induction was measured using fluorescent BODIPY-labeled chloramphenicol (CAM) (Molecular Probes Inc., Eugene, OR) in an 80 μl reaction containing 5 nmol BODIPY-CAM, 0.25 M Tris pH 7.8, 40 nmol acetyl CoA, and either 25 or 50 μg cell protein. After 30 or 60 min the reaction products were extracted with ethyl acetate, spotted on silica thin layer chromatography plates and developed in 90% chloroform, 10% methanol. The images of the fluorescent plates, evenly illuminated with two black-light-blue 15 watt bulbs (predominantly 365 nm UVA; General Electric, Cleveland OH), were digitized by a Star I charge coupled device (CCD) digitizing camera (Photometrics Inc., Tucson, AZ) and stored as tagged image format (TIF) computer files. The reaction products were quantitated from the digitized values for each fluorescent spot on the plate using QUANTISCAN ver. 1.1 software (Biosoft Ltd., Cambridge UK). Each assay contained a positive control and acetylated BODIPY-CAM standards. CAT activity was calculated from the input nmol CAM and the fraction of acetylated products (corrected for background in the substrate). For the positive control, the production of acetylated CAM was linearly related to the input purified CAT enzyme activity up to 45% of the CAM acetylated by 62.5 units

CAT (0.68% ± 0.02% CAM acetylated per unit CAT enzyme, correlation coefficient = 0.99).

In this system UV efficiently induces CAT gene expression in XPHIV-II cells. The addition either of liposomes contain two lipophilic antioxidants, i.e. probucol and vitamin E, or liposomes containing a lipophilic and a hydrophilic antioxidant, i.e. vitamin E and vitamin C, reduced UV induction of viral transcription (Figure 1). The reduction was in a dose-dependent manner.

Of particular note is the extremely low concentrations of antioxidants in the cell culture media delivered by the liposomes needed to reduce HIV activation. Specifically, as shown in Figure 1, an inhibition of 30% of total activation was achieved at dilutions of 20-fold or greater of the liposomes (a maximum of 250 µl of liposomes in 5 ml). Thus, in the case of vitamin E and C liposomes, inhibition of HIV activation was achieved at a vitamin E concentration of 3 µg/ml (0.0003% w/v) and a vitamin C concentration of 28 µg/ml (0.0028% w/v). In the case of vitamin E and probucol liposomes, inhibition was achieved at a vitamin E concentration of 4 µg/ml (0.0004% w/v) and a probucol concentration of 9 µg/ml (0.0009% w/v).

Example 3

Inhibition of UV-induction of HIV in Transgenic Mice by Topical Administration of Antioxidant Liposomes

The ability of antioxidant liposomes to inhibit HIV induction was tested in the HIV transgenic mouse model described by John Morrey, Samuel Bourn, Thomas Bunch, Robert Sidwell and Craig Rosen, "HIV-1 LTR Activation Model: Evaluation of Various Agents in Skin of Transgenic Mice", in the Journal of Acquired Immune Deficiency Syndromes, volume 5, pages 1195-1203, 1992; Morrey J, Bourn S, Bunch T, Jackson M, Sidwells R, Barrows L, Daynes R, Rosen C: In vivo activation of human immunodeficiency virus type 1 long terminal repeat by UV

type A (UV-A) plus psoralen and UV-B light in the skin of transgenic mice. Journal of Virology, 65:5045-5051, 1991.

Lotions were prepared by mixing a 10% (w/v) solution of the hydrogel CARBOPOL-941 in sterile, distilled, demineralized water, and neutralized with triethanolamine. A final lotion was prepared by diluting the hydrogel solution to 2.2% in phosphate buffered saline with 0.55% PHENONIP as a preservative and mixing 11 volumes of this solution with one volume of antioxidant liposomes, prepared as described above. The resulting vitamin E and vitamin C lotion contained 2% hydrogel, 5 μg/ml vitamin E and 47 μg/ml vitamin C. The resulting vitamin E and probucol lotion contained 2% hydrogel, 7 μg/ml vitamin E and 15.5 μg/ml probucol. Control lotions were prepared similarly using empty liposomes, prepared as described above but without the addition of any antioxidants, or by using placebo liposomes, also prepared as described above without the addition of any antioxidants but substituting a heat inactivated DNA repair enzyme, i.e., T4 endonuclease V, for vitamin C in the aqueous phase. An additional control lotion was prepared by dissolving the vitamin E and C liposomes with 25 mM octylglucopyranoside prior to preparation of the liposome lotion.

Hair was removed from the backs of Avertin-anesthetized mice by gently shaving with hair clippers and depilation with a brief treatment of NAIR hair remover (Carter Products, NY, NY). The antioxidant liposomes in hydrogel lotions were topically applied. After 30 min to allow absorption of the lotion, the mice while anesthetized were irradiated with 400 J/m^2 UV-B. The liposome lotions were reapplied at 10 min and 4 hrs post-irradiation, and approximately 24 hours later, HIV gene activation was measured. Activation of the HIV was measured by comparing luciferase reporter gene expression in UV-irradiated sites to unirradiated sites on the same mouse. The method for measuring the reporter gene is

described in Morrey et al., Journal of Acquired Immune Deficiency Syndromes, supra. Relative activation was measured by comparing gene activation in placebo treated skin (liposomes lacking the antioxidants) with activation in active liposome treated skin.

The results are shown in Figure 2, where each bar represents the results from a single transgenic mouse. HIV activation in the placebo treated skin is set to 100%. Liposomal preparations containing vitamin E and C or vitamin E and probucol markedly reduced UV-activation of the HIV-LTR at least 10-fold, to less than 10% of that found in the control. In fact, the antioxidant liposomes slightly reduced baseline levels of HIV-LTR expression in skin of mice not exposed to UV.

The effect at the low antioxidant concentration levels used in these experiments was due to liposome encapsulation of the antioxidants, since dissolution of the liposomes with detergent prior to preparation of the lotion eliminated the effect. The effect was due to the antioxidant activities of the liposomes and not an effect of the lipids themselves, since liposomes which lacked antioxidants or liposomes which had antioxidants which had lost their activity had no effect on induction of HIV.

Although preferred and other embodiments of the invention have been described herein, other embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

What is claimed is:

1. A method for treating an individual infected with a virus, said virus being inducible by reactive oxygen species, said method comprising topically administering at least one antioxidant to the infected individual in an amount effective to reduce induction of said virus by damage to the individual's skin.

2. The method of Claim 1 wherein the at least one antioxidant is selected from the group consisting of α-tocopherol, ascorbic acid, probucol, and mixtures thereof.

3. The method of Claim 1 wherein the at least one antioxidant is incorporated in liposomes.

4. The method of Claim 3 wherein the at least one antioxidant is selected from the group consisting of α-tocopherol, ascorbic acid, probucol, and mixtures thereof.

5. The method of Claim 3 wherein the at least one antioxidant comprises at least one lipophilic antioxidant and at least one hydrophilic antioxidant.

6. The method of Claim 5 wherein the at least one lipophilic antioxidant comprises α-tocopherol and the at least one hydrophilic antioxidant comprises ascorbic acid.

7. The method of Claim 1 wherein the virus is a human immunodeficiency virus.

8. The method of Claim 1 wherein the damage to the individual's skin is caused by UV radiation.

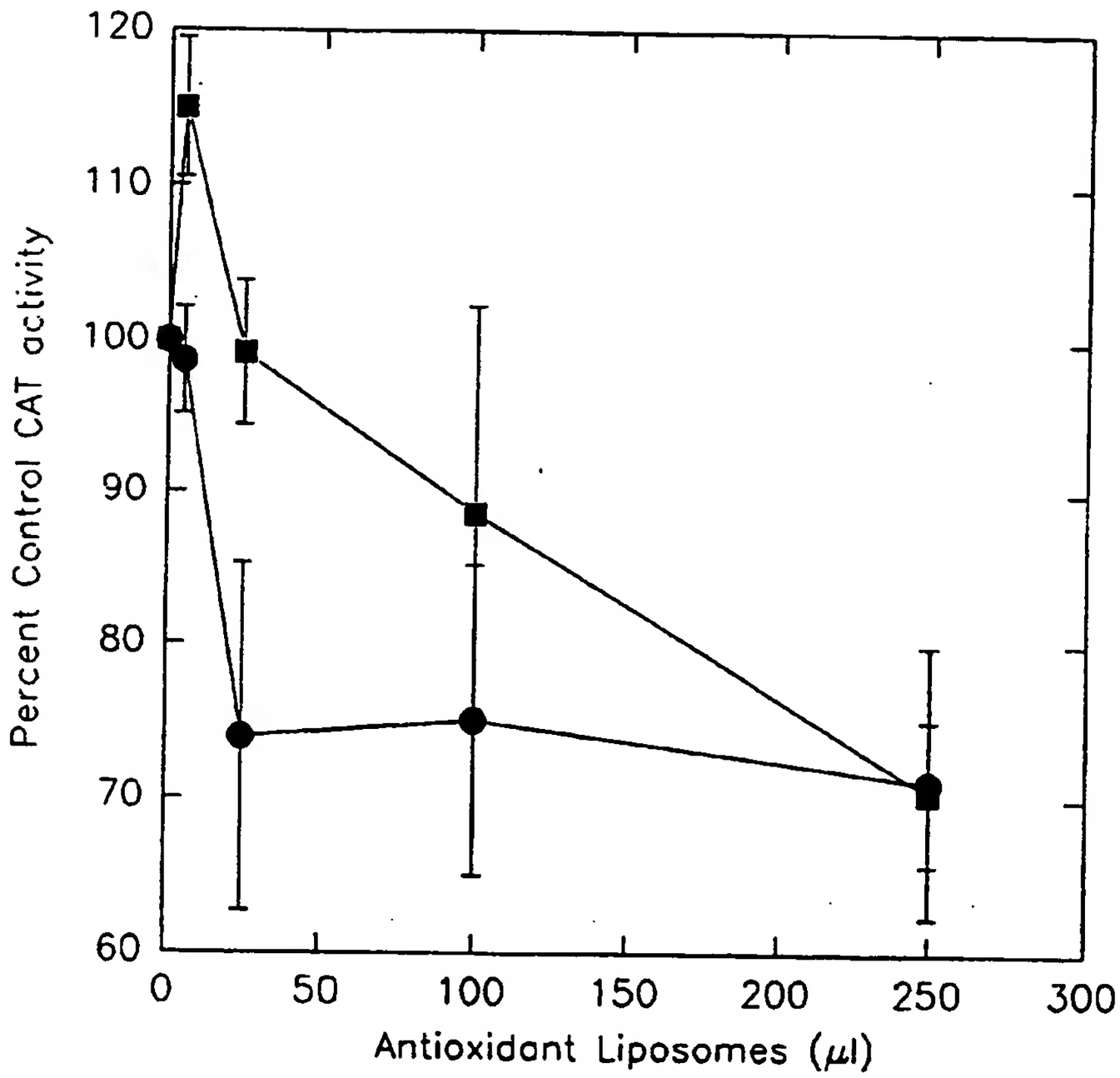
120
110
100
90
80
70
60
Percent Control CAT activity
0
50
100
150
200
250
300
Antioxidant Liposomes (μl)

FIG. 1

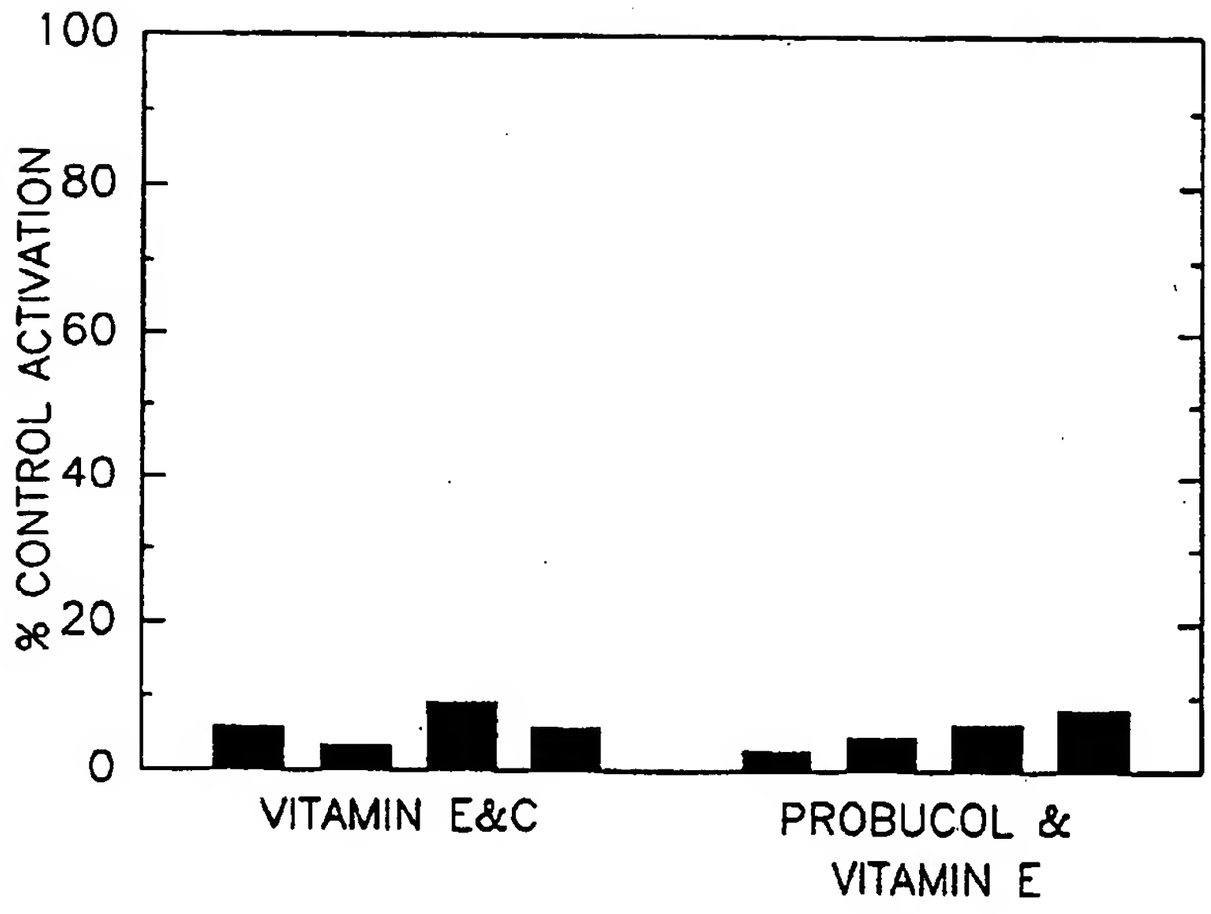
100
80
60
40
20
0
% CONTROL ACTIVATION
VITAMIN E&C
PROBUCOL &
VITAMIN E

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03326

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 9/127
US CL :424/450

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

APS: ANTIOXIDANT?, ANTIVIRAL, TOCOPHEROL?, ASCORBIC ACID, PROBUCOL, LIPOSOME?

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,114,957 (HENDLER) 19 May 1992, column 3, lines 1-18 and claim 5.	1-4, 7
Y	Proc. Natl. Acad. Sci. USA, Volume 87, September 1990, pages 7245-7249, (HARAKEH), "Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells" (See the Abstract).	1-2, 7-8
Y	LETTERS TO NATURE, Volume 333, 05 May 1988, pages 78-81, (VALERIE) "Activation of human immunodeficiency virus type I by DNA damage in human cells" (See the Abstract).	1-2, 7-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 MAY 1994

Date of mailing of the international search report

JUL 05 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03326

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Photodermatology, Photoimmunology and Photomedicine, Volume 7, 1990, pages 56-62, (BISSETT) "Photoprotective effect of superoxide-scavenging antioxidants against ultraviolet radiation-induced chronic skin damage in the hairless mouse" (See the Abstract).	1-2, 7-8
Y	Vitamin E: Biochemistry and Health Implications, Volume 570 of the Annals of the New York Academy of Sciences, 26 December 1989 (PELLE), "In Vitro Model to Assess Alpha-T ocopherol Efficacy" (See page 491, second paragraph).	3-4
Y	VITAMIN E IN HEALTH AND DISEASE, Volume 200, 1992, pages 271-276, (PACKER), "Interactions among Antioxidants in Health and Disease: Vitamin E and Its Redox Cycle", See page 274).	5-6